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The epithelial-mesenchymal transition protein periostin correlates with grading of neuroendocrine lung tumors

Inaugural-Dissertation
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1 Abstract

Background: Among neuroendocrine tumours (NET) of the lung, typical (TC) and atypical (AC) carcinoids represent the low, small cell lung carcinomas (SCLC) and large cell neuroendocrine carcinomas (LCNEC) the high grade spectrum. The N-glycoprotein periostin (POSTN) was recently found to be upregulated in both tumour cell cytoplasm and peritumoural stroma of non-small cell lung carcinomas (NSCLC) in the context of epithelial-mesenchymal transition (EMT). This study aimed at investigating periostin protein expression in NET in these respective compartments.

Patients and Methods: Between 1993 and 2007, a retrospective multicenter cohort of 192 patients with surgically resected NET or diagnosed as such at autopsy was investigated on a tissue microarray (TMA) by immunohistochemistry (IHC). Periostin immunoreactivity was semi-quantitatively scored in the cytoplasm of tumour epithelia and in the peritumoural stroma. Data was correlated with neuroendocrine (NE) categories, markers of neuroendocrine (synaptophysin, chromogranin A) or lung (TTF-1) differentiation, and the tumour proliferation rate Mib-1.

Results: The cohort consisted of 58 TC, 42 AC, 32 LCNEC and 60 SCLC. Concerning neuroendocrine differentiation, chromogranin A was less tightly associated with it than synaptophysin.

Cytosolic periostin expression was higher in carcinoids than in SCLC or LCNEC ($p < 0.001$). There was no significant difference between POSTN expression in tumour cells and overall survival rate ($p = 0.139$). No (grade 0) or weak (grade 1-2) stromal staining with POSTN was detected in more than half of typical (70.7 %) and atypical carcinoids (54.8 %), whereas strong (grade 5-6) staining was observed in LCNEC (56.3 %) and SCLC (46.7 %). Thus high stromal expression of periostin was associated with higher tumour grade and the SCLC and LCNEC categories, respectively, as well as higher pTNM and therefore correlated with a decreased survival rate (p-values <0.05). Furthermore, besides proliferation, a correlation between high TTF-1 expression and worse prognosis was seen in high-grade NET.

Conclusion: Aggressive clinical behaviour of SCLC and LCNEC corresponds not only with high proliferation rate and high expression of TTF-1 but also with the formation of a prominent desmoplastic stroma containing the EMT protein periostin.

2 Introduction

Lung cancer is the most common cancer worldwide and, despite decline, the leading cause of cancer mortality in males. This is largely due to cigarette smoking. Over the past two generations, cancer mortality in females has also increased. It is estimated that approximately 3000 deaths are caused by lung cancer every year in Switzerland.[1]

Nearly all lung cancers are carcinomas and about 80 % of them belong to the non-small cell group (NSCLC), whereas small cell lung cancer (SCLC) constitutes the majority of the rest.[2] In early stages of the disease there are normally no symptoms. Therefore, the diagnosis in most cases will frequently be made too late, and the tumour has often metastasized at the time the patient enters the hospital. In 40-50 % of patients with NSCLC, at the time of diagnosis, the disease has already advanced to stages IIIb or IV.[3]

Referring to an updated World Health Organization (WHO) classification of lung cancer published in 1999 [4], neuroendocrine lung tumours (NET) are a distinct subset sharing certain morphological, ultrastructural, immunohistochemical, and molecular characteristics. The four major categories of morphologically identifiable NET are low-grade typical carcinoids (TC), intermediate-grade atypical carcinoids (AC), and the high-grade small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC), respectively. TC is also called well-differentiated neuroendocrine tumour and AC well-differentiated neuroendocrine carcinoma. They all share neuroendocrine features of varying degrees, detectable by light microscopy.[5] Neuroendocrine immunhistochemical markers are usually best performed as a panel consisting of synaptophysin, chromogranin, and CD56 / NCAM. To date, relatively little is known regarding the genetic changes associated with neuroendocrine lung tumours other than SCLC.[6]

Histologically, typical carcinoids (TC) show a low mitotic rate (less than two per ten high-power fields) and lack of necrosis. Compared to TC, atypical carcinoids (AC) have a larger size with greater architectural disorganization and increased pleomorphism, more frequent mitoses (two to ten per ten high-power fields), tumour necrosis, a higher rate of metastases, and a significantly reduced survival rate.

According to *Travis et al.*[4-5,7-8], large cell neuroendocrine carcinoma (LCNEC) is a tumour composed of large cells characterised by a light microscopic neuroendocrine (NE) appearance such as organoid nesting, trabecular pattern, rosette formation, and basaloid palisading with a high nuclear to cytoplasmic ratio. A frequent presence of prominent macronucleoli, a high mitotic rate (more than 10 mitoses per 10 high power fields), and abundant necrosis is also seen. LCNEC is distinguished from the small cell type (SCLC)

primarily by the nuclear size: If nuclei are 3 times the diameter of a small resting lymphocyte, equal to > 30 micrometer, LCNEC is favoured. Further, a constellation of other criteria, which include larger cell size, abundant cytoplasm, prominent nucleoli, vesicular or coarse chromatin, polygonal shape, and less prominent nuclear moulding is also taken into account.

On the other hand, tumour cells of SCLC have a small round or fusiform shape with salt and pepper chromatin and scant cytoplasm. They grow in sheets or nests with frequent necrosis and their nucleoli are inconspicuous or absent. Decisive for the correct classification of a large cell carcinoma as LCNEC is the detection of at least one immunohistochemical marker or an electron microscopic characterization.[5,8] Several reports suggest that the prognosis of LCNEC is poor, with a variable overall 5-year survival rate ranging from 13% to 45%.[9-10] Another study confirmed that LCNEC shows almost the same prognosis as SCLC,[11] apart from displaying similar histopathologic patterns and common risk factors. Currently, patients with limited SCLC have a 5-year survival rate of 21 %.[12] According to several recent studies, LCNEC responds to cisplatin-based chemotherapeutic regimens similar to those used for SCLC.[13-15] Yet, optimum treatment for LCNEC remains undefined.[16] In many centres a strategy of surgery followed by adjuvant chemotherapy is performed.

Ki-67 (Kiel-67), a non-histone protein, is a DNA-binding nuclear protein expressed throughout the cell cycle in proliferating cells, but not in quiescent (G0) cells. It is detected by the antibody Mib-1 (mitotic labelling index). It has been used to distinguish growing from non-growing cells.[17-19] In small crushed biopsies Ki-67 staining can be helpful to separate TC or AC from high-grade LCNEC or SCLC, which have very high proliferation rates.[20-22]

Thyroid transcription factor-1 (TTF-1) is a nuclear protein playing an important role in the development and morphogenesis of thyroid, lung, and ventral forebrain.[23] In normal adult tissues it is expressed in follicular cells of the thyroid, in Clara cells, type 2 pneumocytes of the lung, and in pituicytes.[23-25] The lung epithelia surface is globally separated into a TTF-1 positive peripheral (alveolar) and a TTF-1 negative central (bronchial) compartment. Therefore, the expression of TTF-1 in tumours deriving from the thyroid, lungs, and neurohypophysis is familiar.

Epithelial-mesenchymal transition (EMT) is defined as transition of epithelial cells into cells with mesenchymal characteristics.[26] During embryogenesis, epithelial cells may lose their epithelial characteristics by dissolving their cell contacts and down-regulating adhesion molecules such as E-cadherin. This permits cells to migrate and pass the basement membrane. Upon reaching their destination they can differentiate into different cells or re-differentiate into epithelial cells (mesenchymal-epithelial transition, MET). A prerequisite for the migration is a polarization of the cell. Moreover, the EMT is associated with cancer

progression and metastasis. Many studies in animals and cell culture experiments have demonstrated that carcinoma cells can acquire a mesenchymal phenotype and express mesenchymal markers (EMT proteins) such as α-SMA (alpha-smooth muscle actin), FSP1 (fibroblast-specific protein 1), vimentin, and desmin.[27] Particularly interesting among these EMT proteins is periostin (POSTN), a gene encoding a secreted 90 kDa protein showing sequence similarity to the insect adhesion molecule fasciclin I.[28] This protein has been suggested to function as a cell adhesion molecule for preosteoblasts and to participate in osteoblast recruitment, attachment, and spreading.[29-30]

The main goal of this work was to analyze the epithelial-mesenchymal transition protein periostin (POSTN) expression in different NE neoplasms and stromal tissue of the lung by tissue microarray (TMA) immunohistochemistry, using a polyclonal anti-POSTN antibody, and its correlation with established histopathological and prognostic factors.

2.1 Objective target

This work was carried out with the following objectives:

1. Production of a tissue microarray with NET of the lung.
2. Acquisition of pathological and clinical patient data.
3. Immunohistochemical detection and analysis of expression of Ki-67, neuroendocrine markers (synaptophysin, chromogranin A), TTF-1 and POSTN.
4. Statistical analysis of pathological parameters and of expression of the named markers.

3 Materials and methods

3.1 Patients and tissue collection

For the time period of 1993 to 2007 a total of 192 patients with surgically resected (n = 183) or autopsy diagnosed (n = 9) neuroendocrine tumours of the lung were investigated. Concerning SCLC, the data set is thus restricted to patients having limited, operable disease. They were retrieved from the computer databases of the *University Hospital* (n = 90) and the *Triemli Hospital in Zurich* (n = 29), as well as the *Technical University of Munich* (n = 73). The study was examined and accredited by the *Institutional Ethical Review Board of the University Hospital of Zurich* under reference number *StV 29-2009*. Because of insufficient material, 29 patients were excluded from the investigation. Additional information such as age, gender, and tumour size were gathered from pathology reports. Tumour stage, grade, and histological subtype were identified according to the TNM criteria, or as recommended by the *UICC*.[31] Surgical and autopsy samples were processed by formalin-fixation and paraffin-embedding according to the guidelines of the *Swiss* and the *German Society of Pathology*. Survival data from 164 patients was collected from the *Cancer Registry Zurich* (*Dr. med. Dimitri Korol*) and *Munich* (*Prof. Dr. med. Aurel Perren*). One hundred patients (52 %) were men and 92 (48 %) were women. The mean age was 57 years, ranging from 15 to 85 years. A tumour size with a mean diameter of 2.9 cm, ranging from 0.5 to 10 cm, was seen in 170 patients. Table 1 shows an overview of the pathological and clinical parameters of the neuroendocrine lung tumours. In 19 patients, the tumour stage and nodal status could not be determined. Furthermore, survival data from 28 patients were missing.

Table 1. NET cohort of the lung. Pathological and clinical parameters.

		Tissue Samples	
		n	**%**
Total		192	100
Histology	SCLC	60	31.3
	TC	58	30.2
	AC	42	21.9
	LCNEC	32	16.7
Tumour stage	pT1	104	60.1
	pT2	55	31.8
	pT3	7	4
	pT4	7	4
Nodal status	pNx	19	9.9
	pN0	126	65.6
	pN1	25	13
	pN2	20	10.4
	pN3	2	1
Metastases	cM0	166	94.3
	cM1	10	5.7
Grading	G1	95	49.5
	G2	5	2.6
	G3	92	47.9
Location			
Side	right	93	53.1
	left	81	46.3
	both	1	0.6
Bronchus	main	8	9.3
	upper lobe	36	41.9
	middle lobe	15	17.4
	lower lobe	20	23.3
	> 1	7	8.1
Lobe	upper lobe	77	45.8
	middle lobe	23	13.7
	lower lobe	54	32.1
	> 1	14	8.3
Survival	alive	89	54.3
	dead	75	45.7

3.2 Construction of Tissue Microarray

According to a method established by *Kononen et al.*,[32] a tissue microarray (TMA) was set up in order to enable a high-throughput molecular profiling of tumour specimens. The configuration of each tumour tissue and its coordinates were stored in a punch and picture file (see Figure 1). HE-stained slides of all tumours were reviewed and representative areas were selected. These were areas with characteristic histomorphology of the individual tumour. A total of 384 tumour tissue cylinders with a diameter of 0.6 mm were punched out from those areas of the corresponding paraffin blocks. From each tumour, two tissue cylinders were taken. Additionally, 16 punches of different control tissues, including normal tissue of the lung and palatine tonsil as well as neuroendocrine tumour tissue of the portio uteri, ileocoecal resectate, and appendix were collected. The complete set, comprising a total of 400 tissue cylinders, was precisely arrayed into a recipient paraffin block using a custom-made, semi-automatic tissue microarray device (Beecher Instruments, Sun Prairie, WI, USA). The device and an overview of the TMA block are displayed in Figure 2.

	x 1	x 2	x 3	x 4	x 5	x 6	x 7	x 8	x 1	x 2	x 3	x 4	x 5	x 6	x 7	x 8	x 1	x 2	x 3	x 4	x 5	x 6	x 7	x 8
y 1	1	2	3	4	5	6	7	8	137	138	139	140	141	142	143	144	273	274	275	276	277	278	279	280
y 2	9	10	11	12	13	14	15	16	145	146	147	148	149	150	151	152	281	282	283	284	285	286	287	288
y 3	17	18	19	20	21	22	23	24	153	154	155	156	157	158	159	160	289	290	291	292	293	294	295	296
y 4	25	26	27	28	29	30	31	32	161	162	163	164	165	166	167	168	297	298	299	300	301	302	303	304
y 5	33	34	35	36	37	38	39	40	169	170	171	172	173	174	175	176	305	306	307	308	309	310	311	312
y 6	41	42	43	44	45	46	47	48	177	178	179	180	181	182	183	184	313	314	315	316	317	318	319	320
y 7	49	50	51	52	53	54	55	56	185	186	187	188	189	190	191	192	321	322	323	324	325	326	327	328
y 8	57	58	59	60	61	62	63	64	193	194	195	196	197	198	199	200	329	330	331	332	333	334	335	336
y 9	65	66	67	68	69	70	71	72	201	202	203	204	205	206	207	208	337	338	339	340	341	342	343	344
y 10	73	74	75	76	77	78	79	80	209	210	211	212	213	214	x	x	345	346	347	348	349	350	351	352
y 11	81	82	83	84	85	86	87	88	217	218	219	220	221	222	223	224	353	354	355	356	357	358	359	360
y 12	89	90	91	92	93	94	95	96	225	226	227	228	229	230	231	232	361	362	363	364	365	366	367	368
y 13	97	98	99	100	101	102	103	104	233	234	235	236	237	238	239	240	369	370	371	372	373	374	375	376
y 14	105	106	107	108	109	110	111	112	241	242	243	244	245	246	247	248	377	378	379	380	381	382	383	384
y 15	113	114	115	116	117	118	119	120	249	250	251	252	253	254	255	256	385	386	387	388	389	390	391	392
y 16	121	122	123	124	125	126	127	128	257	258	259	260	261	262	263	264	393	394	395	396	397	398	399	400
y 17	129	130	131	132	133	134	135	136	265	266	267	268	269	270	271	272								

SCLC **TC** **AC** **LCNEC** **normal** **other** **x** **error**

Figure 1. Picture file of TMA.

A

B

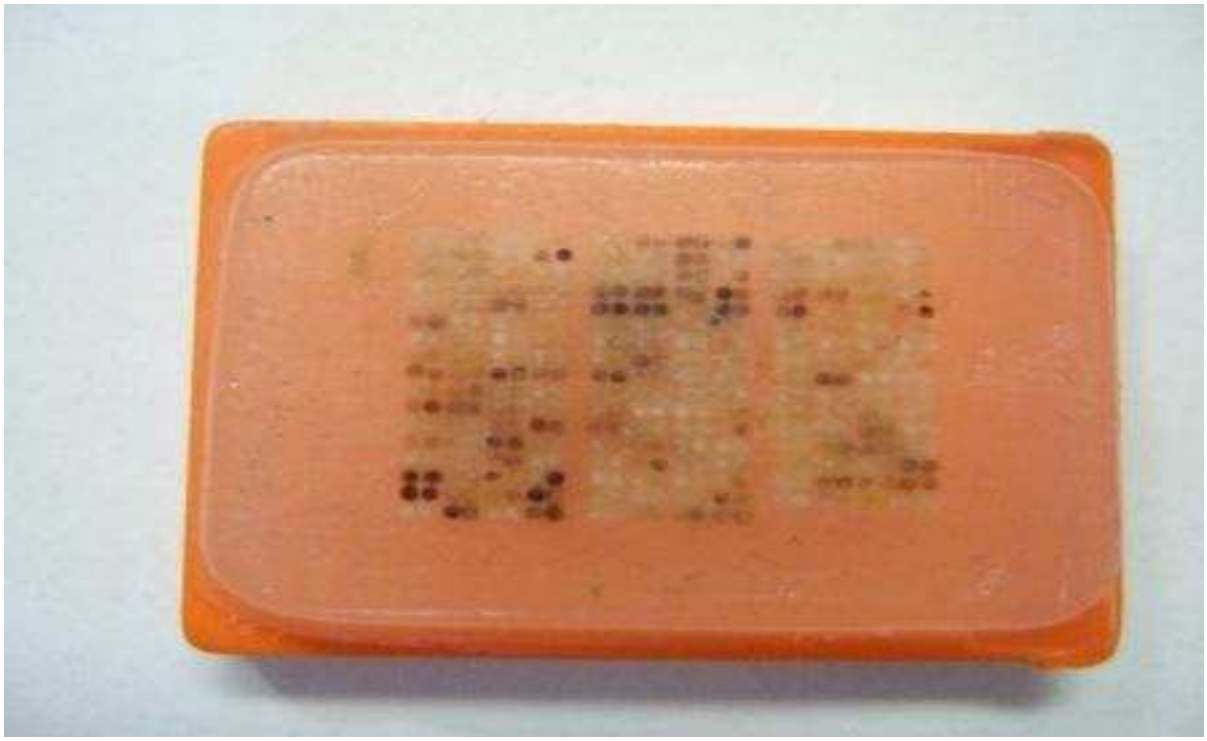

Figure 2. A: Tissue array device for the construction of the TMA.

B: Overview of the tissue microarray block.

3.3 Immunohistochemistry

For immunohistochemical analysis, 4-µm-thick paraffin sections were cut from the TMA block and mounted on silane-coated glass slides. After incubation at 37°C overnight, deparaffinization in xylene, and rehydration through graded ethanol series, the sections were stained with a *avidin-biotin-peroxidase complex method*[33] using a *Ventana* automat (*Ventana Medical Systems, Tucson, AZ*) for Ki-67, synaptophysin (syn), chromogranin A (cg A), and TTF-1. For POSTN, we used a *Bond* automat (*Vision Biosystems, Melbourne Australia*) according to the following protocol: after boiling in Tris pH 8, containing buffer H2 for 30 min, the slide was incubated with the rabbit polyclonal anti-POSTN antibody for 30 minutes at room temperature. Detection by means of secondary antibodies was performed with *Ultraview Amp* (*Ventana*) and *Refine-DAB* (*Vision Biosystems*) kits. The five antibodies, their dilutions, and the type of pretreatment are listed in Table 2.

Table 2. Antibodies.

Protein	Antibody	Clone	Producer	Dilution	Pretreatment
Ki-67	Mouse	Mib-1	DAKO	1:20	*CC1m**
Syn	Mouse	27G12	Ventana	prediluted	*CC1m**
Cg A	Mouse	LK2H10	Ventana	prediluted	*CC1m**
TTF-1	Mouse	8G7G3/1	DAKO	1:50	*CC1m**
POSTN	Rabbit	polyclonal	BioVendor	1:500	None

**CC1* = Cell conditioning solution 1 (*Ventana Medical Systems, Inc.*): prediluted solution, which is used for the pretreatment for automatic immunostaining of tissue samples on the *Ventana BenchMark*® device.

m = mild cell condition (30 min boiling).

3.4 Interpretation of results

For each core the intensity of immunoreactivity was semi-quantitatively scored on a four tiered scale, with grade 0 equivalent to no staining, grade 1 = weak, grade 2 = moderate, and grade 3 = strong. Epithelial and stromal protein expression of POSTN was scored by *Andrej Atanassoff*. Ki-67 was scored for frequency ranging from 0 to 100 % and intensity from 0 to 4 % equivalent to grade 0, 5-9 % = grade 1, 10-20 % = grade 2, and > 20 % = grade 3 as well as syn, cg A, and TTF-1 for intensity only (0 to 3) by *Andrej Atanassoff* and *Christian Oehlschlegel*. The scores of both cores were summed up to range from 0 to 6 resulting in grade 0, no staining, grade 1-2, weak, grade 3-4, moderate, and grade 5-6 equivalent to strong staining.

3.5 Statistical analysis

Of the 221 original patients, 29 were excluded since no adequate tumour tissue was available. Of the 192 remaining patients, 28 were removed from the survival analysis due to lack of data. Survival calculations were thus computed on a total of 164 patients. The correlation between various pathological-anatomical or immunohistochemical parameters was investigated by multi-field for multiple groups or non-parametric *Kruskal-Wallis* test. For all investigations with respect to patient prognosis, the overall survival was chosen as the endpoint. For calculation of the survival time, living patients were evaluated as "censored".

The illustration of the cumulative survival curves was performed by the method of *Kaplan-Meier*. A *log rank* test was used for the statistical assessment of differences between cumulative survival curves. Statistical analyses were performed using the statistical software package *SPSS/PASW 16.0.0* (*SPSS Inc., Chicago, IL, USA*). *P*-values < 0.05 were considered statistically significant in all analyses.

4 Results

4.1 Pathological-anatomical findings

After completion of the TMA paraffin block, a 4-µm thin section was stained with Hematoxylin- Eosin (HE). All tumours were reviewed for morphology and compared with the data in the punch file. Figure 3 shows a digital photo of an HE-stained TMA section. Examples of histomorphological types of NET of the lung are illustrated in Figures 4-7.

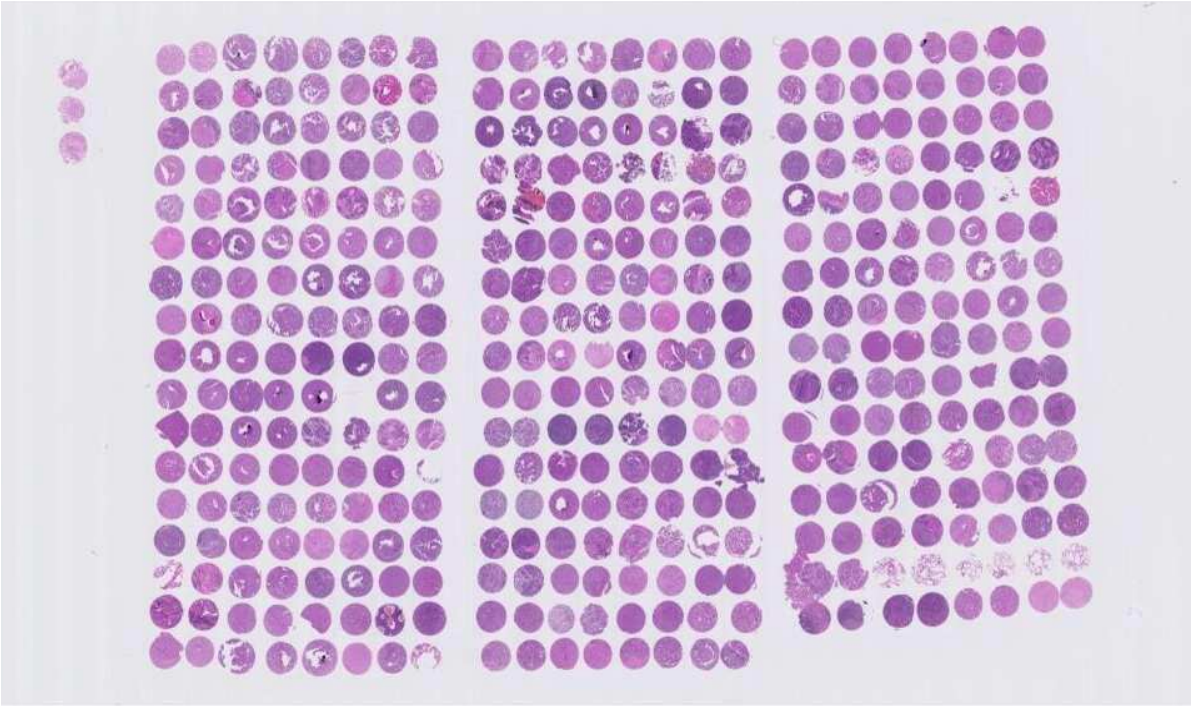

Figure 3. HE-stained TMA section. Original size: 2 x 1.3 cm.

A a

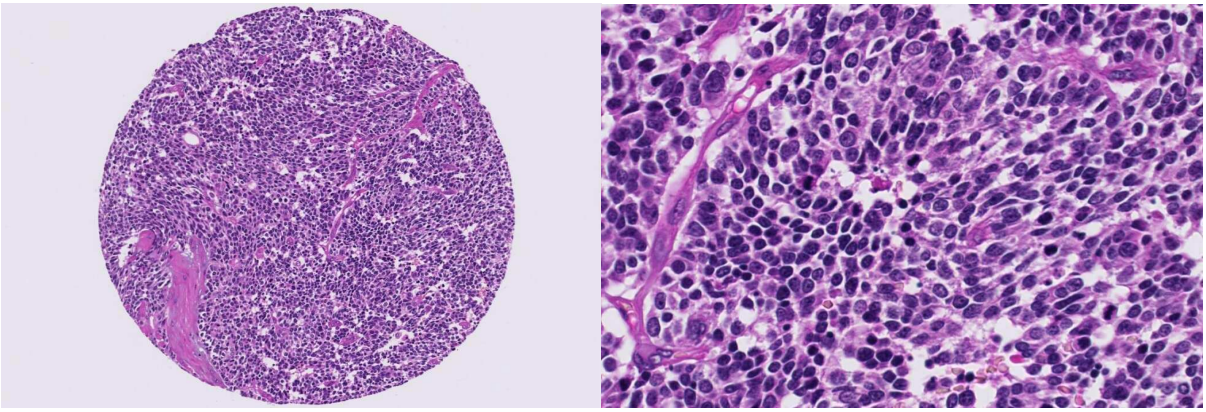

Figure 4. A: SCLC, magnification: 10x; a: magnification: 40x.

B b

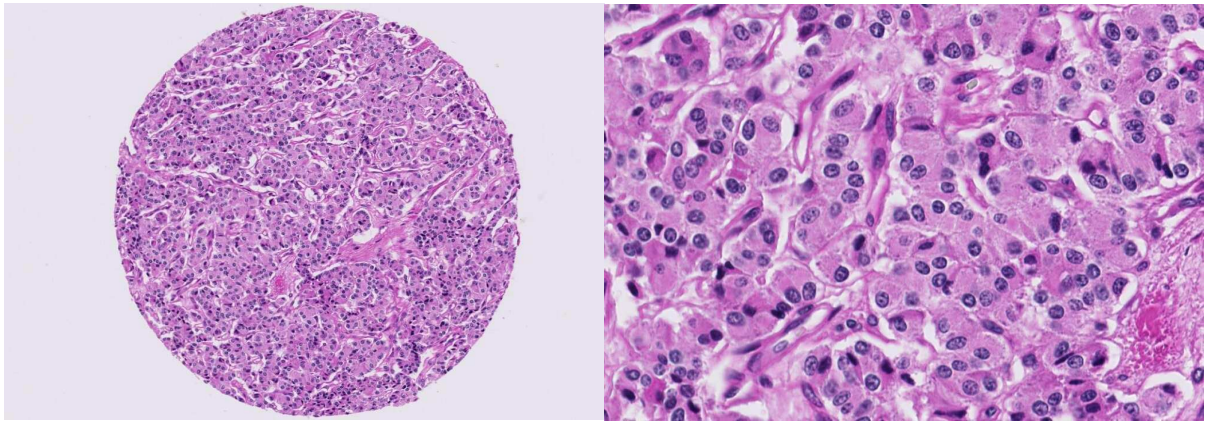

Figure 5. B: TC, magnification: 10x; b: magnification: 40x.

C c

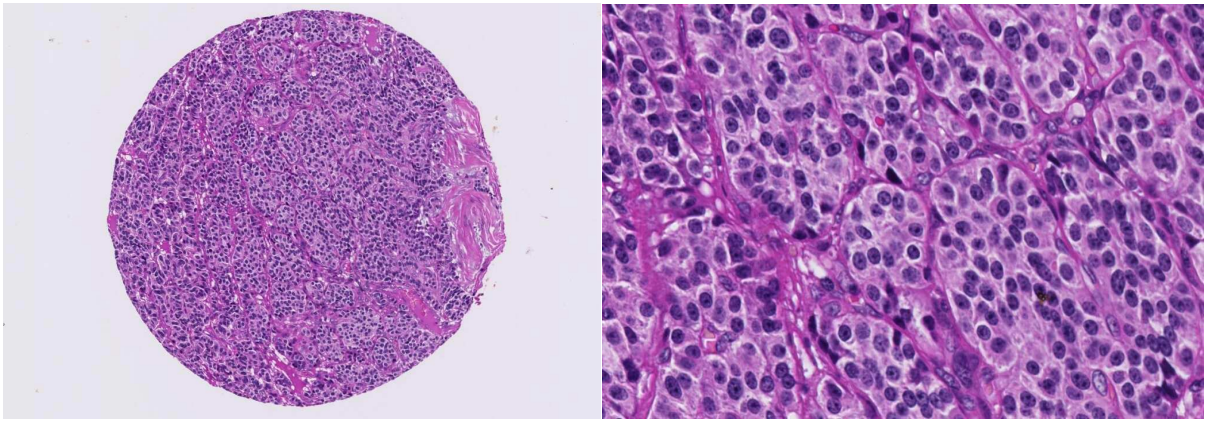

Figure 6. C: AC, magnification: 10x; c: magnification: 40x.

D d

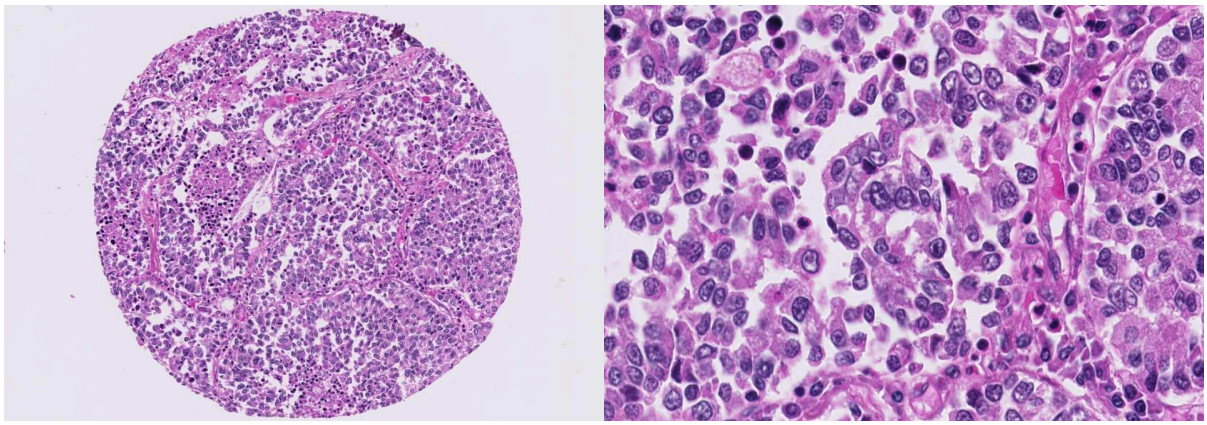

Figure 7. D: LCNEC, magnification: 10x; d: magnification: 40x.

The tumour stage, nodal status, the presence of metastases, and grading were closely associated with the patients` prognosis ($p < 0.001$). Significant differences were also found with the individual types of NET of the lung ($p < 0.001$). The correlations between these parameters and overall survival regardless of the tumour type are shown in Figures 8-11. Figure 12 illustrates overall survival considering well versus poorly differentiated neuroendocrine lung tumours. Table 3 indicates the 5-year survival rate of the four tumour types.

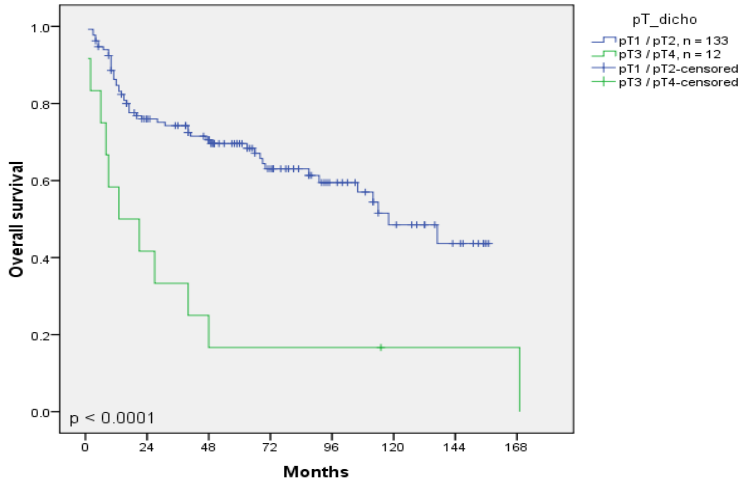

Figure 8. Tumour stage and overall survival.

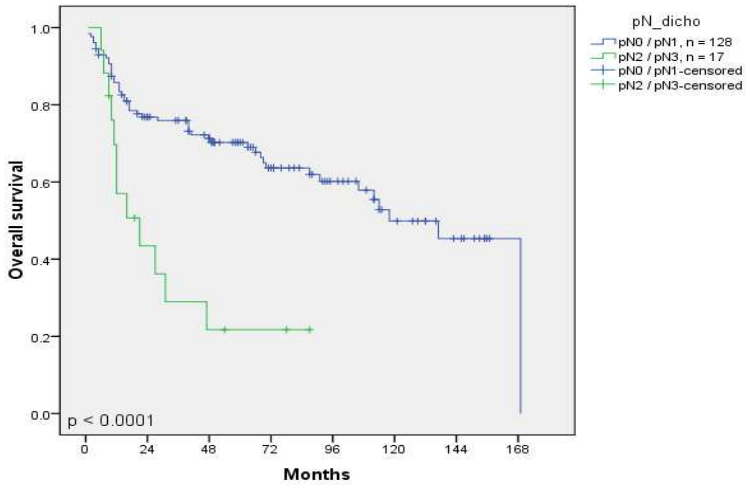

Figure 9. Nodal status and overall survival.

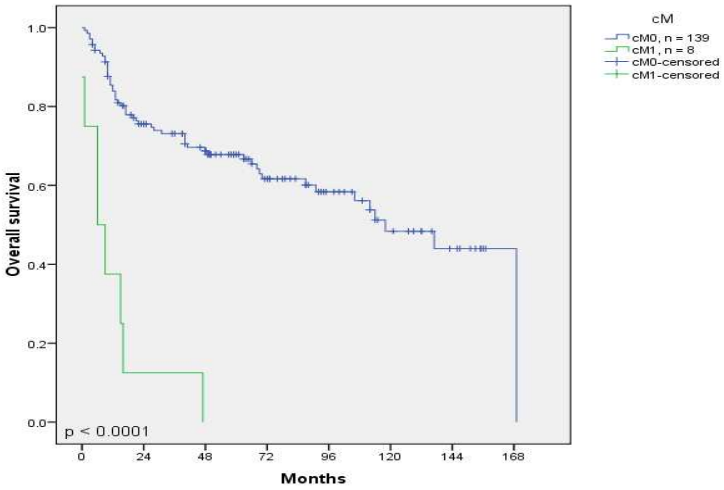

Figure 10. Metastases and overall survival.

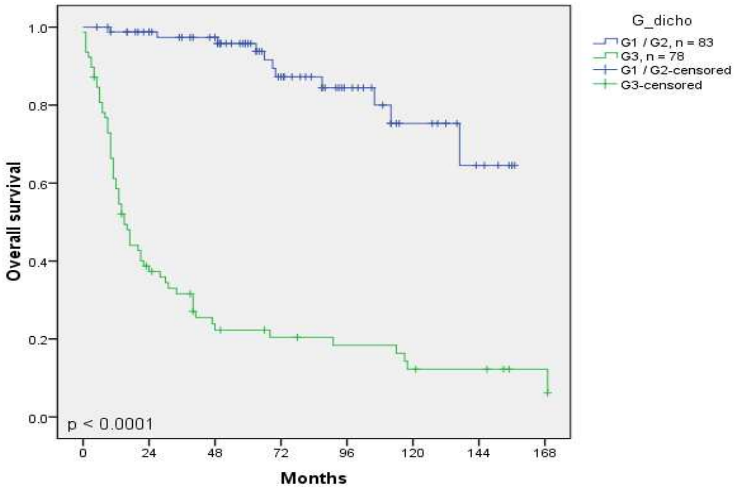

Figure 11. Grading and overall survival.

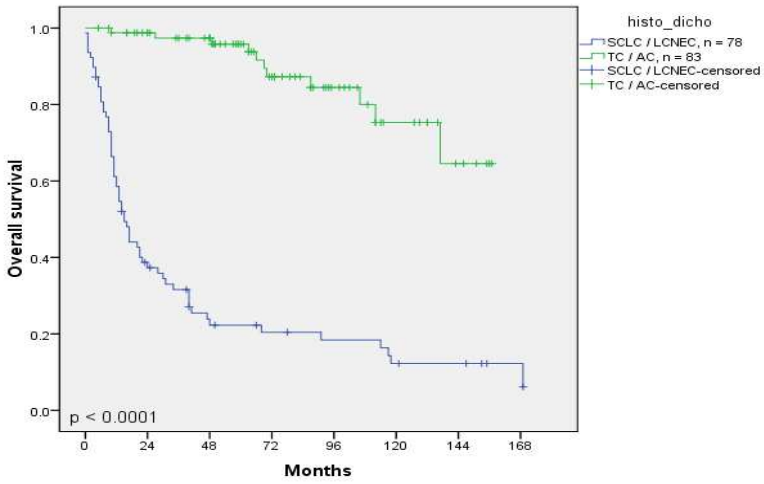

Figure 12. Well versus poorly differentiated NET of the lung and overall survival.

Table 3. Overall 5-year survival according to tumour type.

Type	Overall 5-year survival
SCLC	21.0 %
TC	97.2 %
AC	93.5 %
LCNEC	24.0 %

4.2 Immunohistochemical findings

In order to determine the correlation between protein expression and pathological parameters, the data obtained from the analysis with the antibodies Ki-67, synaptophysin, chromogranin A, TTF-1 and periostin was compared with the histological type of tumour. On the basis of survival data, it was possible to calculate the clinical significance of individual molecular parameters. The tumour stage, nodal status, the presence of metastases, and grading were also included in the calculations.

4.2.1 Ki-67

Any Ki-67 positivity was observed in 102 of 192 analyzable NET of the lung (53.1 %). A Ki-67 negative AC and a strong Ki-67 positive SCLC is shown in Figure 13. It is known that SCLC and LCNEC have a higher proliferation rate than TC and AC. This is also seen in Table 4. There was a significant positive correlation between strong Ki-67 positivity and high grade NET, higher tumour stage pT3 / pT4, higher nodal stage pN2 / pN3, presence of metastases, and higher grading G3.

A B

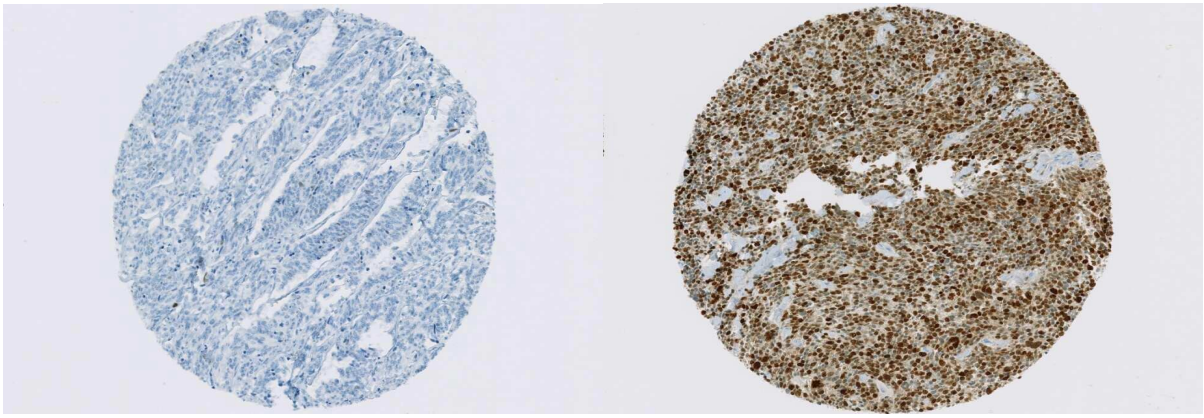

Figure 13. A: Ki-67 negative AC. Magnification: 10x.
B: Strong Ki-67 positive SCLC. Magnification: 10x.

Table 4. Ki-67 expression and histopathological parameters.

			Ki-67 sum intensity scores						
			0	1	2	3	4	5	6
		n	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Histology	SCLC	60	4 (6.7)		1 (1.7)	1 (1.7)	4 (6.7)	6 (10.0)	44 (73.3)
p < 0.001	TC	58	54 (93.1)	3 (5.2)			1 (1.7)		
	AC	42	31 (73.8)	4 (9.5)	2 (4.8)		5 (11.9)		
	LCNEC	32	1 (3.1)		1 (3.1)		2 (6.3)	4 (12.5)	24
(75.0)									
Tumour stage	pT1 / pT2	159	85 (53.5)	7 (4.4)	3 (1.9)	1 (0.6)	8 (5.0)	8 (5.0)	47 (29.6)
p < 0.001	pT3 / pT4	14	1 (7.1)				2 (14.3)		11 (78.6)
Nodal Status	pN0 / pN1	151	82 (54.3)	7 (4.6)	2 (1.3)	1 (0.7)	5 (3.3)	8 (5.3)	46 (30.5)
p < 0.003	pN2 / pN3	22	4 (18.2)		1 (4.5)		5 (22.7)		12 (54.5)
Metastasis	cM0	166	86 (51.8)	7 (4.2)	3 (1.8)		9 (5.4)	8 (4.8)	53 (31.9)
p < 0.001	cM1	10				1 (10.0)	1 (10.0)		8 (80.0)
Grading	G1 / G2	100	85 (85.0)	7 (7.0)	2 (2.0)		6 (6.0)		
p < 0.001	G3	92	5 (5.4)		2 (2.1)	1 (1.1)	6 (6.5)	10 (10.9)	68 (73.9)

The result of the prognostic significance of Ki-67 mitotic labelling index in NET of the lung is shown in Figure 14. A negative (grade 0) or low (grade 1-2) Ki-67 expression had no clinical significance. But a strong (grade 5-6) Ki-67 positivity was significantly associated with worse prognosis ($p < 0.001$).

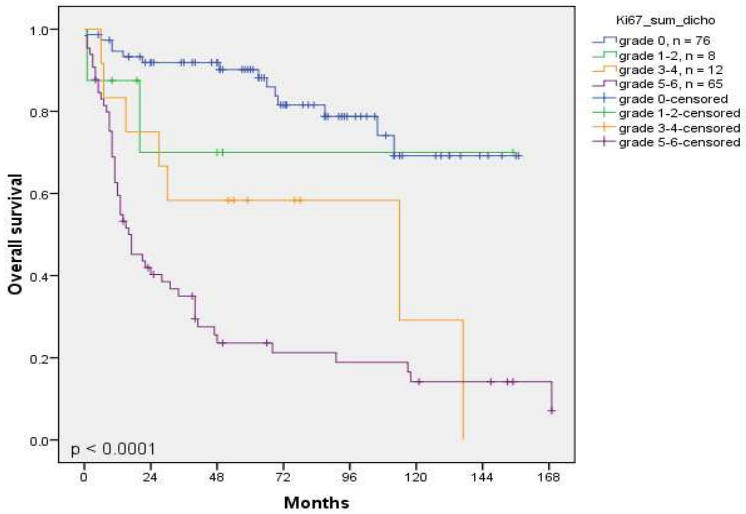

Figure 14. Ki-67 mitotic labelling index and overall survival in lung NET.

4.2.2 Synaptophysin (syn)

Any expression of synaptophysin was detected in a total of 184 of 192 analyzable NET of the lung (95.8 %). Figure 15 illustrates a syn negative LCNEC and a strong syn positive TC. The correlation between syn expression and tumour type is shown in Table 5. There was a significant positive correlation between strong syn positivity and carcinoids, lower tumour stage pT1 / pT2, and lower grading G1 / G2.

A B

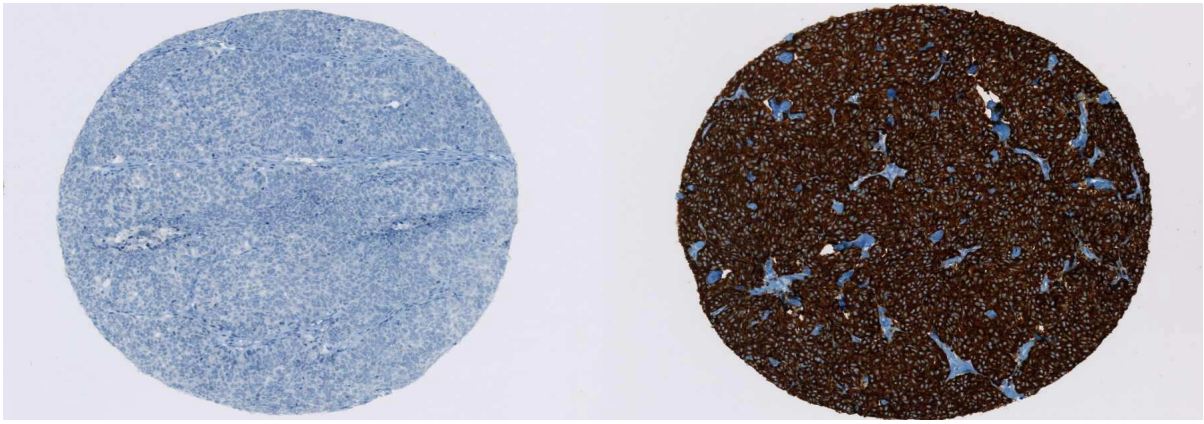

Figure 15. A: Syn negative LCNEC. Magnification: 10x.
B: Strong syn positive TC. Magnification: 10x.

Table 5. Syn expression and histopathological parameters.

			Syn sum intensity scores						
			0	1	2	3	4	5	6
		n	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Histology	SCLC	60	4 (6.7)	7 (11.7)	13 (21.7)	8 (13.3)	5 (8.3)	2 (3.3)	21 (35.0)
p < 0.001	TC	58	1 (1.7)		2 (3.4)	2 (3.4)	2 (3.4)	4 (6.9)	47 (81.0)
	AC	42			2 (4.8)		1 (2.4)		39 (92.9)
	LCNEC	32	3 (9.4)		6 (18.8)	5 (15.6)	8 (25.0)	3 (9.4)	7 (21.9)
Tumour stage	pT1 / pT2	159	6 (3.8)	5 (3.1)	13 (8.2)	13 (8.2)	13 (8.2)	7 (4.4)	102 (64.2)
p = 0.008	pT3 / pT4	14	1 (7.1)		5 (35.7)	1 (7.1)	2 (14.3)	1 (7.1)	4 (28.6)
Nodal Status	pN0 / pN1	151	7 (4.6)	5 (3.3)	14 (9.3)	13 (8.6)	12 (7.9)	8 (5.3)	92 (60.9)
p = 0.785	pN2 / pN3	22			4 (18.2)	1 (4.5)	3 (13.6)		14 (63.6)
Metastasis	cM0	166	7 (4.2)	4 (2.4)	17 (10.2)	13 (7.8)	15 (9.0)	8 (4.8)	102 (61.4)
p = 0.413	cM1	10		1 (10.0)	2 (20.0)	1 (10.0)		1 (10.0)	5 (50.0)
Grading	G1 / G2	100	1 (1.0)		4 (4.0)	2 (2.0)	3 (3.0)	4 (4.0)	86 (86.0)
p < 0.001	G3	92	7 (7.6)	7 (7.6)	19 (20.7)	13 (14.1)	13 (14.1)	5 (5.4)	28 (30.4)

A negative, weak or moderate (grade 3-4) syn expression was significantly associated with poor prognosis ($p < 0.001$). The result of this statistical analysis is shown in Figure 16.

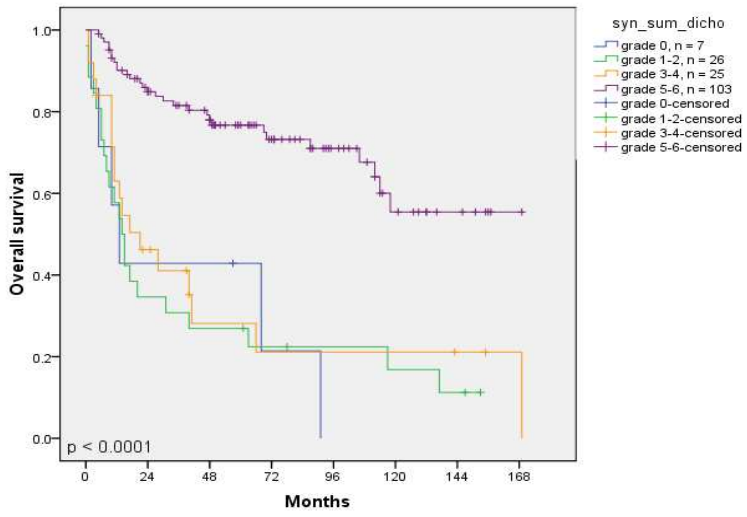

Figure 16. Syn expression and overall survival in lung NET.

4.2.3 Chromogranin A (cg A)

Any chromogranin A expression was observed in 148 of 192 analyzable NET of the lung (77.1%). Figure 17 illustrates examples of a cg A negative SCLC and a strong cg A positive TC. The correlation between the expression of cg A and the tumour type is shown in Table 6. There was a significant positive correlation between strong cg A positivity and carcinoids, lower tumour stage pT1 / pT2, lower nodal stage pN0 / pN1, absence of metastases, and lower grading G1 / G2.

A B

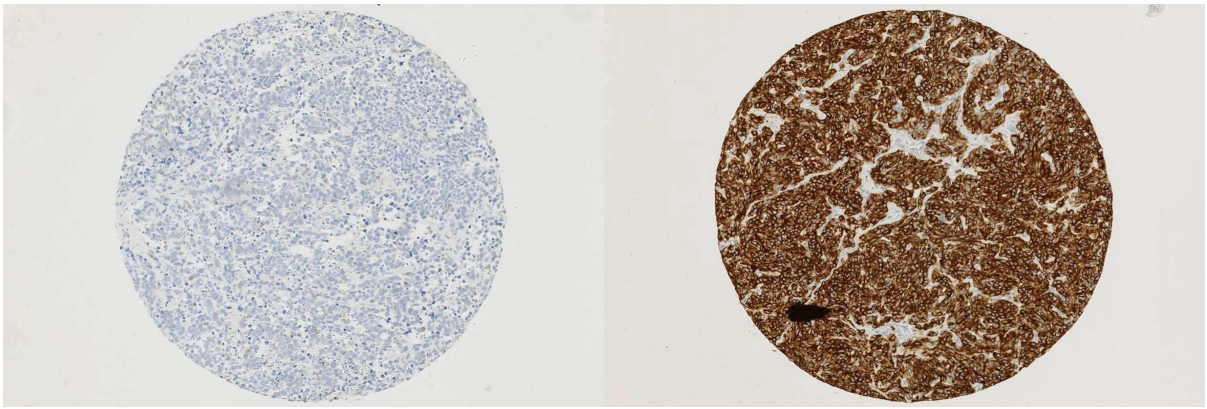

Figure 17. A: Cg A negative SCLC. Magnification: 10x.
B: Strong cg A positive TC. Magnification: 10x.

Table 6. Cg A expression and histopathological parameters.

			Cg A sum intensity scores						
			0	1	2	3	4	5	6
		n	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Histology	SCLC	60	30 (50.0)	4 (6.7)	19 (31.7)	2 (3.3)	5 (8.3)		
p < 0.001	TC	58	1 (1.7)	1 (1.7)	1 (1.7)	2 (3.4)	11 (19.0)	4 (6.9)	38 (65.5)
	AC	42	1 (2.4)		2 (4.8)	2 (4.8)	13 (31.0)	1 (2.4)	23 (54.8)
	LCNEC	32	12 (37.5)	5 (15.6)	10 (31.3)	3 (9.4)	1 (3.1)		1 (3.1)
Tumour stage	pT1 / pT2	159	32 (20.1)	9 (5.7)	17 (10.7)	8 (5.0)	27 (17.0)	5 (3.1)	61 (38.4)
p = 0.002	pT3 / pT4	14	6 (42.9)		7 (50.0)				1 (7.1)
Nodal Status	pN0 / pN1	151	31 (20.5)	7 (4.6)	18 (11.9)	8 (5.3)	24 (15.9)	5 (3.3)	58 (38.4)
p = 0.022	pN2 / pN3	22	7 (31.8)	2 (9.1)	6 (27.2)		3 (13.6)		4 (18.2)
Metastasis	cM0	166	36 (21.7)	9 (5.4)	20 (12.0)	8 (4.8)	26 (15.7)	5 (3.0)	62 (37.3)
p = 0.043	cM1	10	2 (20.0)	1 (10.0)	5 (50.0)		2 (20.0)		
Grading	G1 / G2	100	2 (2.0)	1 (1.0)	3 (3.0)	4 (4.0)	24 (24.0)	5 (5.0)	61 (61.0)
p < 0.001	G3	92	42 (45.7)	9 (9.8)	29 (31.5)	5 (5.4)	6 (6.5)		1 (1.1)

A negative or weak cg A expression was significantly associated with poor prognosis ($p < 0.001$). The result of this statistical analysis is shown in Figure 18.

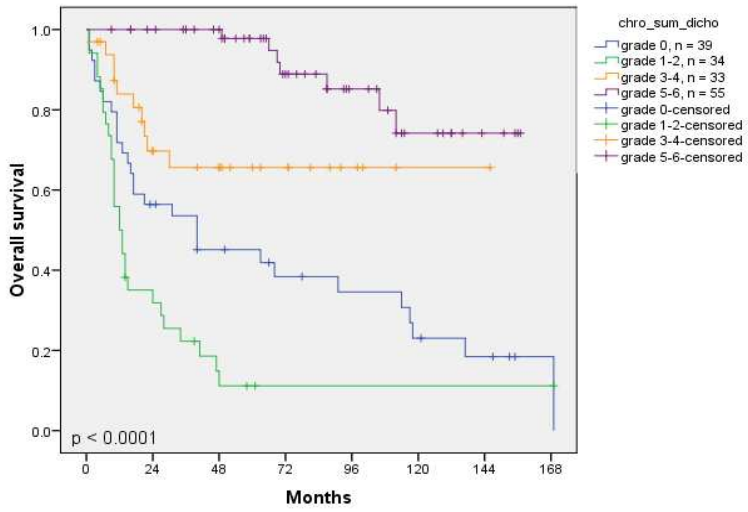

Figure 18. Cg A expression and overall survival in lung NET.

4.2.4 Thyroid transcription factor-1 (TTF-1)

Any TTF-1 expression was detected in a total of 124 of 192 analyzable NET of the lung (64.6 %). Figure 19 illustrates a TTF-1 negative AC and a strong TTF-1 positive LCNEC. The relations between TTF-1 and histopathologic parameters are listed in Table 7. There was a significant positive correlation between strong TTF-1 positivity and high grade NET, presence of metastases, and higher grading G3.

A B

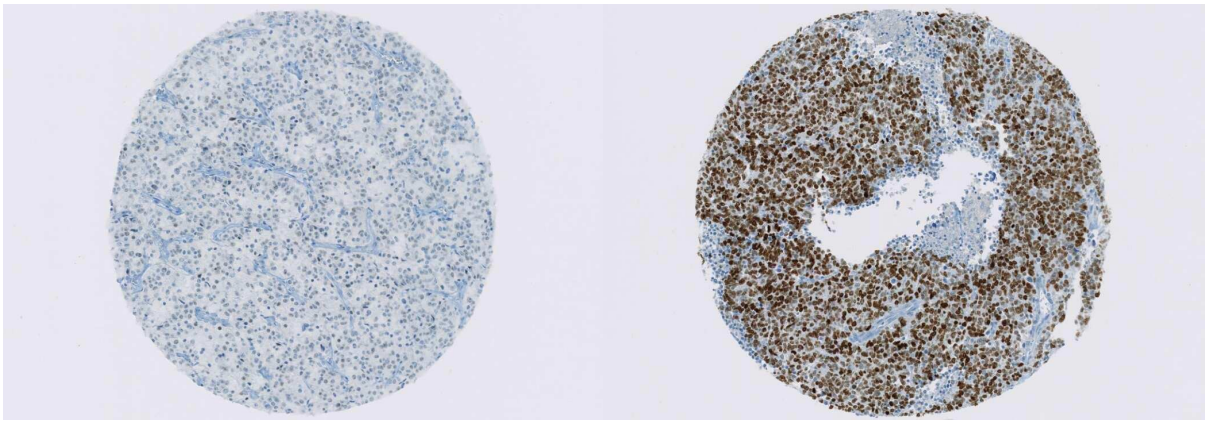

Figure 19. A: TTF-1 negative AC. Magnification: 10x.
B: Strong TTF-1 positive LCNEC. Magnification: 10x.

Table 7. TTF-1 expression and histopathologic parameters.

			TTF-1 sum intensity scores						
			0	1	2	3	4	5	6
		n	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Histology	SCLC	60	12 (20.0)	3 (5.0)	13 (21.7)	5 (8.3)	9 (15.0)	2 (3.3)	16 (26.7)
p < 0.001	TC	58	32 (55.2)	3 (5.2)	18 (31.0)	2 (3.4)	3 (5.2)		
	AC	42	22 (52.4)	4 (9.5)	13 (31.0)	1 (2.4)	2 (4.8)		
	LCNEC	32	2 (6.3)	5 (15.6)	8 (25.0)	3 (9.4)	4 (12.5)	1 (3.1)	9 (28.1)
Tumour stage	pT1 / pT2	159	59 (37.1)	13 (8.2)	45 (28.3)	9 (5.7)	14 (8.8)	1 (0.6)	18 (11.3)
p = 0.192	pT3 / pT4	14	4 (28.6)	1 (7.1)	2 (14.3)	2 (14.3)	1 (7.1)	1 (7.1)	3 (21.4)
Nodal Status	pN0 / pN1	151	58 (38.4)	10 (6.6)	40 (26.5)	11 (7.3)	13 (8.6)	1 (0.7)	18 (11.9)
p = 0.445	pN2 / pN3	22	5 (22.7)	4 (18.2)	7 (31.8)		2 (9.1)	1 (4.5)	3 (13.6)
Metastasis	cM0	166	63 (38.0)	14 (8.4)	43 (25.9)	9 (5.4)	15 (9.0)	2 (1.2)	20 (12.0)
p = 0.006	cM1	10			4 (40.0)	2 (20.0)	1 (10.0)		3 (30.0)
Grading	G1 / G2	100	54 (54.0)	7 (7.0)	31 (31.0)	3 (3.0)	5 (5.0)		
p < 0.001	G3	92	14 (15.2)	8 (8.7)	21 (22.8)	8 (8.7)	13 (14.1)	3 (3.3)	25 (27.2)

The result of the prognostic significance of TTF-1 expression in NET of the lung is shown in Figure 20. A strong (grade 5-6) TTF-1 expression was significantly associated with worse prognosis (p = 0.003).

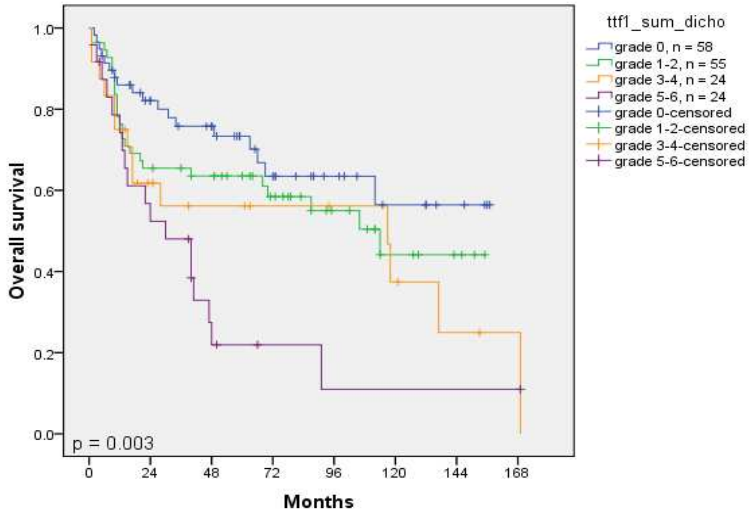

Figure 20. TTF-1 expression and overall survival in lung NET.

4.2.5 Periostin (POSTN)

Any POSTN protein expression in the cytoplasm of tumour epithelia was observed in 183 of 192 analyzable NET of the lung (95.3 %), whereas stromal staining occurred in 165 tumours (85.9 %). A SCLC with POSTN negative cytoplasm and an AC with strong POSTN positive cytoplasm are illustrated in Figure 21a. Figure 21b indicates a TC with POSTN negative peritumoural stroma and a LCNEC with strong POSTN positive peritumoural stroma. The relations between POSTN upregulation in the tumour cell respectively in the peritumoural stroma and the histopathologic parameters are listed in Table 8a and 8b (see also Figure 22). POSTN immunoreactivity in the cytoplasm of carcinoids and LCNEC was higher than in the cytoplasm of SCLC ($p < 0.001$). It was also correlated with lower stage (as a trend, $p = 0.054$), absence of metastases ($p = 0.047$) and lower grade ($p < 0.001$). Inversely, peritumoural periostin expression was higher in SCLC and LCNEC than in carcinoids ($p < 0.001$). Further, high stromal POSTN was correlated with higher stage pT ($p = 0.048$), presence of lymph node ($p = 0.05$, as trend) and organ metastases ($p = 0.006$).

A B

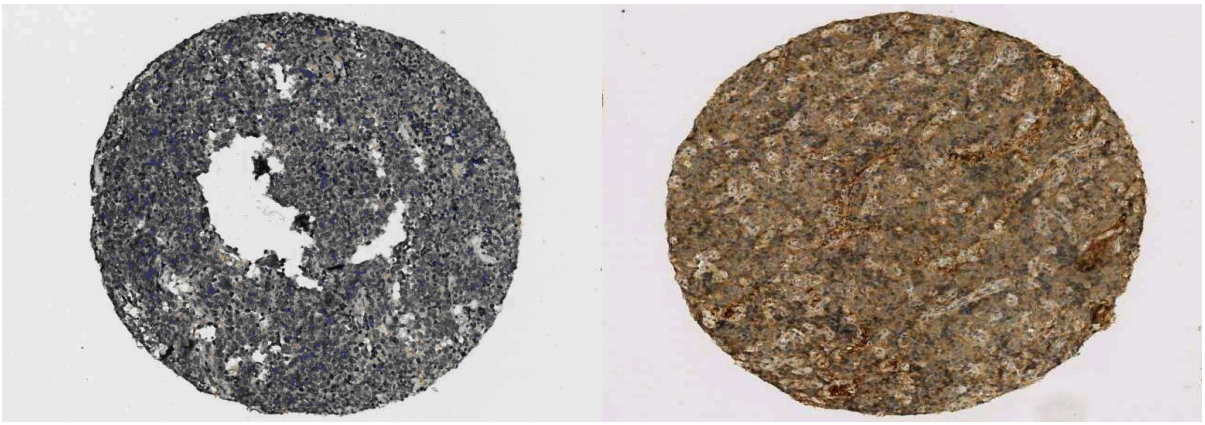

Figure 21a. A: SCLC with POSTN negative cytoplasm. Magnification: 10x.
B: AC with strong POSTN positive cytoplasm. Magnification: 10x.

A B

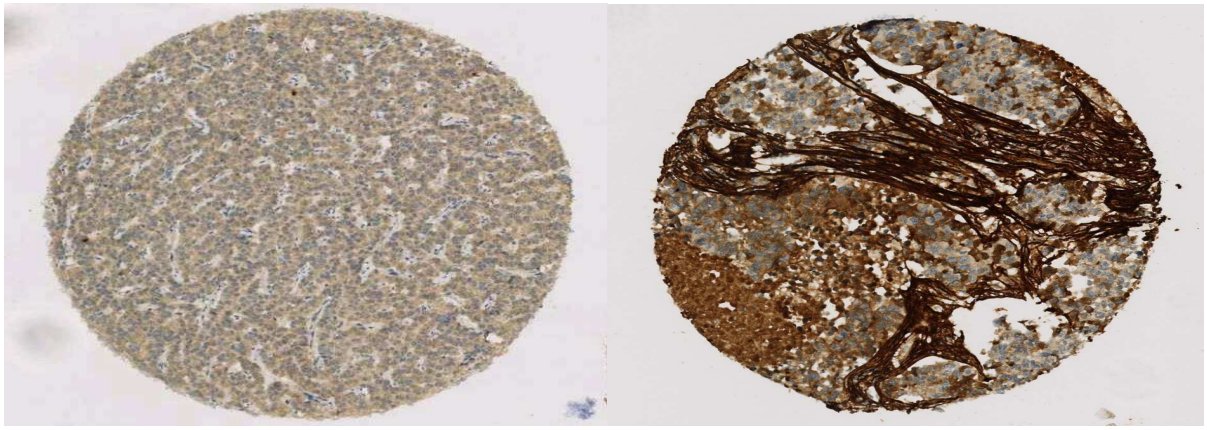

Figure 21b. A: TC with POSTN negative peritumoural stroma. Magnification: 10x.

B: LCNEC with strong POSTN positive peritumoural stroma. Magnification: 10x.

Table 8a. Tumour epithelial expression of POSTN and histopathological parameters.

			POSTN sum intensity scores in tumours						
			0	1	2	3	4	5	6
		n	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Histology	SCLC	60	6 (10.0)	6 (10.0)	16 (26.7)	11 (18.3)	12 (20.0)	2 (3.3)	7 (11.7)
p < 0.001	TC	58	2 (3.4)	2 (3.4)	2 (3.4)	7 (12.1)	17 (29.3)	5 (8.6)	23 (39.7)
	AC	42	1 (2.4)		1 (2.4)	5 (11.9)	12 (28.6)	3 (7.1)	20 (47.6)
	LCNEC	32		1 (3.1)	3 (9.4)	3 (9.4)	9 (28.1)	11 (34.4)	5 (15.6)
Tumour stage	pT1 / pT2	159	6 (3.8)	5 (3.1)	18 (11.3)	18 (11.3)	44 (27.7)	20 (12.6)	48 (30.2)
p = 0.054	pT3 / pT4	14		3 (21.4)	1 (7.1)	4 (28.6)	3 (21.4)	1 (7.1)	2 (14.3)
Nodal Status	pN0 / pN1	151	3 (2.0)	7 (4.6)	16 (10.6)	21 (13.9)	41 (27.2)	18 (11.9)	45 (29.8)
p = 0.302	pN2 / pN3	22	3 (13.6)	1 (4.5)	3 (13.6)	1 (4.5)	6 (27.3)	3 (13.6)	5 (22.7)
Metastasis	cM0	166	6 (3.6)	8 (4.8)	16 (9.6)	20 (12.0)	46 (27.7)	21 (12.7)	49 (29.5)
p = 0.047	cM1	10	1(10)		4 (40.0)	2 (20.0)	1 (10.0)		2 (20.0)
Grading	G1 / G2	100	3 (3.0)	2 (2.0)	3 (3.0)	12 (12.0)	29 (29.0)	8 (8.0)	43 (43.0)
p < 0.001	G3	92	6 (6.5)	7 (7.6)	19 (20.7)	14 (15.2)	21 (22.8)	13 (14.1)	12 (13.0)

Table 8b. POSTN expression in peritumoural stroma and histopathological parameters.

			POSTN sum intensity scores in peritumoural stroma						
			0	1	2	3	4	5	6
		n	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Histology	SCLC	60	1 (1.7)	3 (5.0)	8 (13.3)	10 (16.7)	10 (16.7)	15 (25.0)	13 (21.7)
p < 0.001	TC	58	18 (31.0)	6 (10.3)	17 (29.3)	7 (12.1)	4 (6.9)	5 (8.6)	1 (1.7)
	AC	42	7 (16.7)	7 (16.7)	9 (21.4)	9 (21.4)	5 (11.9)	3 (7.1)	2 (4.8)
	LCNEC	32	1 (3.1)		2 (6.3)	4 (12.5)	7 (21.9)	10 (31.3)	8 (25.0)
Tumour stage	pT1 / pT2	159	26 (16.4)	15 (9.4)	32 (20.1)	25 (15.7)	20 (12.6)	24 (15.1)	17 (10.7)
p = 0.048	pT3 / pT4	14		1 (7.1)	2 (14.3)	2 (14.3)	3 (21.4)	4 (28.6)	2 (14.3)
Nodal Status	pN0 / pN1	151	23 (15.2)	15 (9.9)	33 (21.9)	22 (14.6)	22 (14.6)	21 (13.9)	15 (9.9)
p = 0.05	pN2 / pN3	22	3 (13.6)	1 (4.5)	1 (4.5)	5 (22.7)	1 (4.5)	7 (31.8)	4 (18.2)
Metastasis	cM0	166	26 (15.7)	16 (9.6)	34 (20.5)	26 (15.7)	22 (13.3)	24 (14.5)	18 (10.8)
p = 0.006	cM1	10			1 (10.0)	1 (10.0)	1 (10.0)	5 (50.0)	2 (20.0)
Grading	G1 / G2	100	25 (25.0)	13 (13.0)	26 (26.0)	16 (16.0)	9 (9.0)	8 (8.0)	3 (3.0)
p < 0.001	G3	92	2 (2.2)	3 (3.3)	10 (10.9)	14 (15.2)	17 (18.5)	25 (27.2)	21 (22.8)

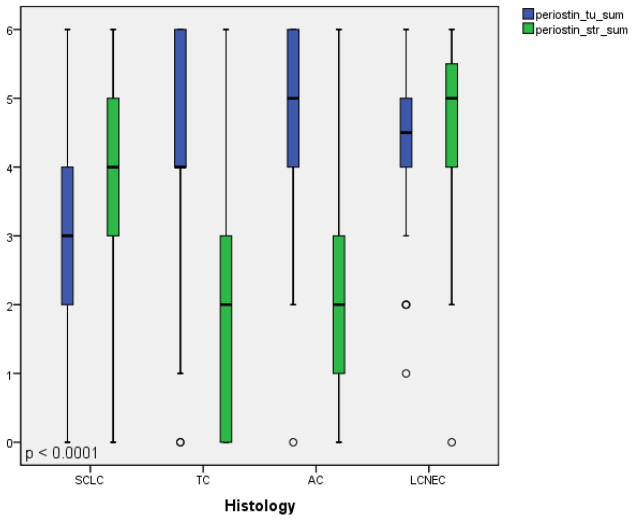

Figure 22. POSTN sum intensity scores in the NET cytoplasm and the surrounding desmoplastic stroma.

As shown in Figure 23a, there was no relevant difference between POSTN expression in epithelial tumour cells and overall survival (p = 0.139). But a strong POSTN positivity in the peritumoural stroma was closely associated with worse prognosis, as seen in Figure 23b (p = 0.003).

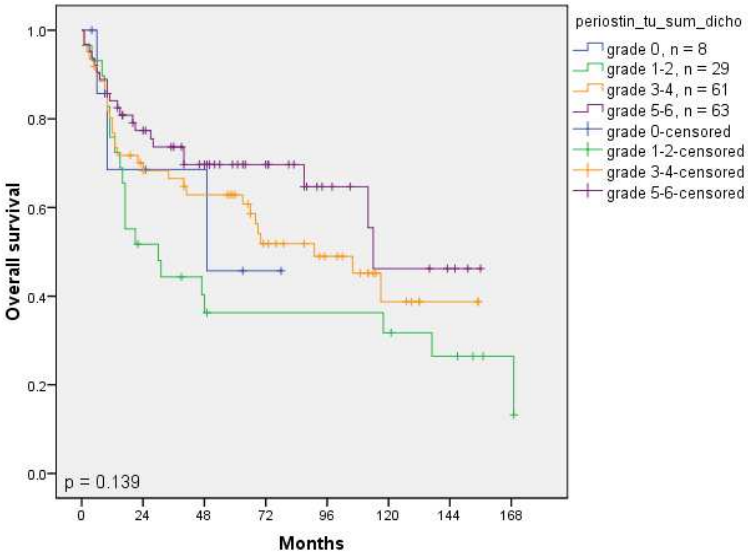

Figure 23a. Epithelial expression of POSTN and overall survival in lung NET.

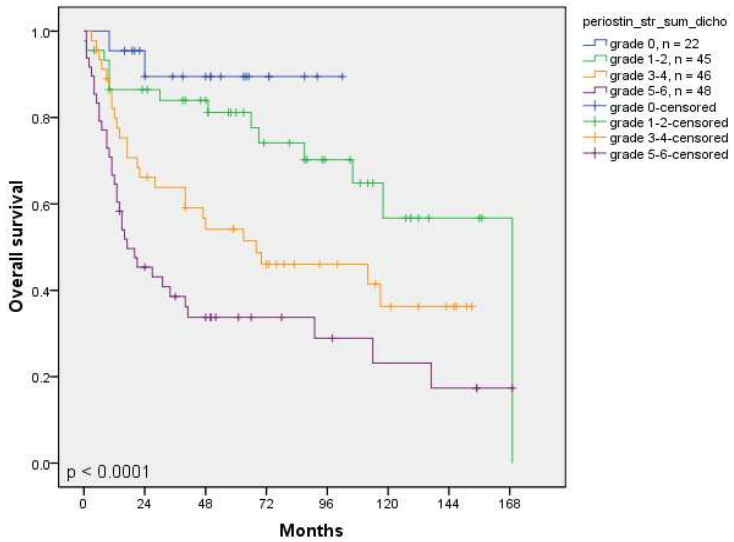

Figure 23b. POSTN expression in peritumoural stroma and overall survival in lung NET.

5 Discussion

Tissue microarrays (TMA) allow efficient analysis of molecular markers in malignant human tumours. Using 0.6 mm punch cores, > 600 different tumours may be scored simultaneously within a short a period of time without damaging or depleting too much tissue. Moreover, the costs for fluid agents used for analysis such as fluorescence in situ hybridization (FISH), RNA in situ hybridization (ISH) or immunohistochemistry (IHC) are significantly reduced. For one TMA section the same reaction volume is administered as needed for a single large section. TMAs also allow full anonymity of the tissue samples with preservation of the collected clinical information and simplify the research cooperation between various institutions. Although it must be kept in mind that a potential limitation of TMA is that small core samples might not be representative of an entire tumour, particularly so in heterogeneous cancers,[34] the use of TMA has the advantage of enabling protein profiling, which probably more closely reflects the biologic characteristics of the tumour cells than does RNA detection.

Besides histomorphological criteria, neuroendocrine (NE) markers (synaptophysin (syn), chromogranin A (cg A)) were helpful to classify all tumours. Rarely, both tumour TMA cores, especially considering cases of SCLC or LCNEC, showed no staining for both syn (SCLC 6.7 %, LCNEC 9.4 %) and cg A (SCLC 50 %, LCNEC 37.5 %). These tumours were nevertheless included in the study, since in the archival reports NE differentiation was verified via positivity for syn or cg A or via an additional marker such as CD56 or NSE. Cg A is generally assumed to be less significant than syn concerning NE differentiation.[35] This was also observable in the present cohort. *Rossi et al.*[36] found the following percentage of immunohistochemical expression in LCNEC for cg A (65%) and syn (53%). *Hiroshima et al.*[37] detected the following percentage of immunohistochemical staining with syn and cg A in SCLC (syn 57 %, cg A 36 %) and LCNEC (syn 77 %, cg A 59 %).

Molecular mechanisms associated with neuroendocrine tumours (NET) of the lung and cancer progression in general remain poorly understood. During the past decade, global gene expression profiling studies on various human cancer types, mainly relying on cDNA microarray technology, led to the identification of new candidate genes involved in cancer progression. In this study, special interest was given to the epithelial-mesenchymal transition (EMT) protein periostin (POSTN). Up-regulation of this protein was observed in various cancers such as breast[38], colon[39], head and neck[40] and oral[41] cancer. Previous work in non-small cell lung carcinoma (NSCLC) by *Sasaki et al.*[42] showed that POSTN immunoreactivity in serum could be determined using sandwich ELISA (enzyme linked immunosorbent assay).

Furthermore, POSTN mRNA was detected at the border of epithelial tumour cells and surrounding stroma.[43] Recently, *Soltermann et al.*[44] studied the prognostic relevance of this EMT indicator protein POSTN in NSCLC. POSTN was found to correlate with squamous cell histotype, higher pT, larger size and higher grade. Further, it was a prognosticator for decreased progression-free survival on univariate analysis (p-value 0.007). In general, POSTN protein seems to play an important role in invasion, angiogenesis, and metastasis, as demonstrated by using tumour cell lines and mice models. However, another study[45] has shown that the expression of POSTN is significantly downregulated in human bladder cancer tissues and that POSTN suppresses cell invasiveness and metastasis of cancer cells, thus rather acting as a tumour suppressor.

The major objective of this work was to comprehensively investigate both epithelial and stromal periostin (POSTN) expression in all 4 categories of NET of the lung and its correlation with histopathological parameters. Epithelial and stromal staining with any distinct POSTN intensity (grade 1-6) was seen in all four neuroendocrine (NE) categories. Epithelial POSTN expression with strong staining was found as follows: small cell lung carcinoma (SCLC) 15.0 %, typical carcinoid (TC) 48.3 %, atypical carcinoid (AC) 54.8 %, large cell neuroendocrine carcinoma (LCNEC) 50.0 %. POSTN immunoreactivity in the cytoplasm of TC/AC and LCNEC was higher than in the cytoplasm of SCLC. However, this could be primarily due to the fact that SCLCs have very scant cytoplasm. No relation with overall survival was observed. The functional significance of cytosolic POSTN expression in tumour epithelia still remains unclear. It does not seem to be an effect of passive influx, since mRNA is detectable by ISH.[38,42-43] Rather, tumour cells at the invasion front up-regulated expression of EMT proteins themselves in order to facilitate movement into their own newly formed desmoplastic stroma. No or weak stromal staining with POSTN was detected in over half of typical (70.7 %) and atypical carcinoids (54.8 %), whereas strong staining was observed with frequencies of 56.3 % in LCNEC and 46.7 % in SCLC. In other words, strong POSTN positivity in the peritumoural stroma correlates with high-grade NET of the lung ($p < 0.001$). Both tumour entities thus have a highly active desmoplastic stroma. As expected and seen in Fig. 22, LCNEC has somewhat more stromal POSTN expression than SCLC. Large cell carcinomas generally have more prominent desmoplastic stroma than SCLCs, which present more “lymphoid tissue-like” with extensive necrosis. Some authors reported POSTN upregulation in about half of the NSCLC tumours[46] and a slight increase in POSTN serum levels from NSCLC patients.[42] Higher POSTN serum levels were also associated with higher T- or N-stage in SCLC patients;[43] but other authors found a down-regulation of POSTN gene transcription in SCLC tumours by in situ mRNA hybridization.[47] Furthermore, peritumoural

up-regulation of POSTN in SCLC and LCNEC is prognostically significant concerning overall survival ($p = 0.003$). In addition to its prognostic significance.

A strong expression of the proliferation marker Ki-67 is associated with tumour stage, nodal status or grading in various types of tumours. Regardless of classic pathological parameters, presence of Ki-67 is also prognostically relevant in tumours such as bladder[48-49] or prostate cancer,[50] and is therefore routinely used in cancer diagnosis. Often, the Ki-67 labeling index (Mib-1) was adducted for the evaluation of Ki-67. It indicates the percentage of tumour cells expressing Ki-67 in a specific tumour site. An elevated Mib-1 is often associated with late tumour stage or high-grade of differentiation. However, it is difficult to find an optimal threshold, which allows statements about the prognosis. Depending on the analyzed tumour type, but also according to staining intensity and evaluation of each pathologist, the thresholds of Mib-1 vary considerably. To work around this problem, the scores of both cores of each tumour were added up to a sum of 0 to 6, which simplified the statistical calculations in this study.

Based on the present results and early studies [7,13,51] LCNEC have a higher proliferation activity than TC and AC. Thus, Ki-67 helps to distinguish low-grade from high-grade NE lung tumours. Furthermore, Ki-67 expression in SCLC and LCNEC correlates with pathological parameters such as tumour stage, nodal status, presence of metastasis or grading. A strong Ki-67 positivity in high-grade NET is significantly associated with poor survival ($p < 0.0001$).

In lung tumours, thyroid transcription factor-1 (TTF-1) is mainly expressed in adenocarcinoma and high-grade NET (SCLC and LCNEC), but it is absent in squamous cell carcinoma (SCC).[52-55] The frequency of TTF-1 expression in SCLC (80 %), but not in LCNEC (93.7 %), found in this study are similar to the results published by *Folpe et al.*[56] and *Sturm et al.*[57] But there were also found frequencies of TTF-1 in 57 % of SCLC and 24 % of LCNEC.[37] A possible explanation for the high expression of TTF-1 in LCNEC in this study is that different antibody clones and IHC protocols are used. Immunoreactivity of TTF-1 is known to be clone-dependent. Further, these tumours could be combined with other histological tumour types such as SCLC or adenocarcinomas. A strong TTF-1 positivity was observed in high-grade NET, which was significantly associated with poor prognosis ($p = 0.003$). As far as concerning SCLC, this was as well seen by *Hiroshima et al.*[37] This cohort study reported that 44.8 % of TC and 47.6 % of AC were TTF-1 positive. Some authors suggested a high specificity and sensitivity for TTF-1 immunohistochemistry following detection of pulmonary carcinoids[58] while others did not find any TTF-1 expression in typical and atypical carcinoids.[29,37-38,57,59-60] This discrepancy could be related to differences in

immunohistochemical techniques or in assessing the threshold of positivity. However, TTF-1 could be a helpful marker in differentiating pulmonary from gastrointestinal carcinoids.[61-62]

In general, the study shows that the possible heterogeneity within a tumour does not substantially affect the results of TMA analysis. Although the small tissue samples of TMA in individual cases are not always representative of the entire tumour, this error factor due to the large number of well-selected tumours and tumour areas is balanced, so that relevant clinical-pathological correlations remain detectable.[32,63-66]

The growing number of TMA-literature data shows that the TMA technology has become an indispensable tool in cancer research. It is expected that the use of TMA in particular to evaluate the clinical relevance of molecular changes is much easier. Furthermore, collected tumour collectives in large standardized prospective studies could be transferred to a TMA format, from which new opportunities would arise for cooperation, validation of research results and the achievement of an international consensus. For example, a new promising prognostic factor in all existing analog collectives could be verified within a short period of time. Such a decision was recently taken for e.g. the EML4-ALK translocation in NSCLC by ETOP (European Thoracic Oncology Platform). In order to obtain consistent FISH data, all TMAs must have the same design, consisting of 4 cores of 1 mm diameter each per tumour.

In conclusion, this immunohistochemical investigation describes the differential expression in both tumour epithelial and peritumoural stroma of the EMT protein periostin in the 4 major categories of lung NET and its correlation to histopathological and prognostic factors. Aggressive clinical behaviour of SCLC and LCNEC corresponds not only with high mitotic proliferation rate but also with the formation of a prominent desmoplastic stroma with high metabolic activity.

6 References

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This work is dedicated to my beloved parents.

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